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Defective intracellular transport and processing of OA1 is a major cause of ocular albinism type 1

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Ocular albinism type 1 (OA1) is an X-linked disorder mainly characterized by a severe reduction of visual acuity, hypopigmentation of the retina and the presence of macromelanosomes in the skin and eyes. Various types of mutation have been identified within the *OA1* gene in patients with the disorder, including several missense mutations of unknown functional significance. In order to shed light into the molecular pathogenesis of ocular albinism and possibly define critical functional domains within the OA1 protein, we characterized 19 independent missense mutations with respect to processing and subcellular distribution on expression in COS-7 cells. Our analysis indicates the presence of at least two distinct biochemical defects associated with the different missense mutations. Eleven of the nineteen OA1 mutants (~60%) were retained in the endoplasmic reticulum, showing defecNStive intracellular transport and glycosylation, consistent with protein misfolding. The remaining eight of the nineteen OA1 mutants (~40%) displayed sorting and processing behaviours indistinguishable from those of the wild-type protein. Consistent with our recent findings that OA1 represents a novel type of intracellular G protein-coupled receptor (GPCR), we found that most of these latter mutations cluster within the second and third cytosolic loops, two regions that in canonical GPCRs are known to be critical for their downstream signaling, including G protein-coupling and effector activation. The biochemical analysis of OA1 mutations performed in this study provides important insights into the structure–function relationships of the OA1 protein and implies protein misfolding as a major pathogenic mechanism in OA1.

INTRODUCTION

Ocular albinism type 1 (OA1; X-linked ocular albinism of the Nettleship–Falls type; MIM 300500) is the most common form of ocular albinism, with an estimated prevalence of about 1:50 000 (1). This disorder is transmitted as an X-linked trait with affected males showing the complete phenotype and heterozygous females showing only minor retinal and cutaneous signs of the disease. Affected males with ocular albinism have substantial reduction of visual acuity and manifest horizontal and occasionally rotary nystagmus, strabismus and marked photophobia. Ophthalmologic examination reveals iris translucency, foveal hypoplasia and hypopigmentation of the retina. As with other types of albinism, patients with ocular albinism have misrouting of the optic tracts, resulting in loss of stereoscopic vision (1). Cutaneous changes are mild in ocular albinism: hypopigmentation of the skin may be noted only by comparison with unaffected siblings (1). Ultrastructural examination of skin melanocytes and of the retinal pigment epithelium typically reveals the presence of structural abnormalities of melanosomes, mainly represented by macromelanosomes, suggesting an underlying defect in the biogenesis of these organelles (2). Consistently, the recent data that we obtained with an *Oa1* mouse knockout strongly support the idea that the protein product of the *OA1* gene is required for proper maturation of melanosomes (3).

We previously isolated the human and mouse *OA1* genes and showed that they encode predicted proteins of 404 and 405 amino acids, respectively, display several putative transmembrane domains and are exclusively expressed in the pigment cells of the skin and eye (melanocytes and retinal pigment epithelium) (4,5). By using polyclonal antibodies we identified the endogenous OA1 protein in normal human melanocytes as a 60 kDa glycoprotein and a 48–45 kDa doublet, corresponding to unglycosylated precursor polypeptides. Immunofluorescence and immunogold analyses in human melanocytes demonstrated that the endogenous OA1 is localized to the melanosomal membrane, consistent with its possible involvement in melano-

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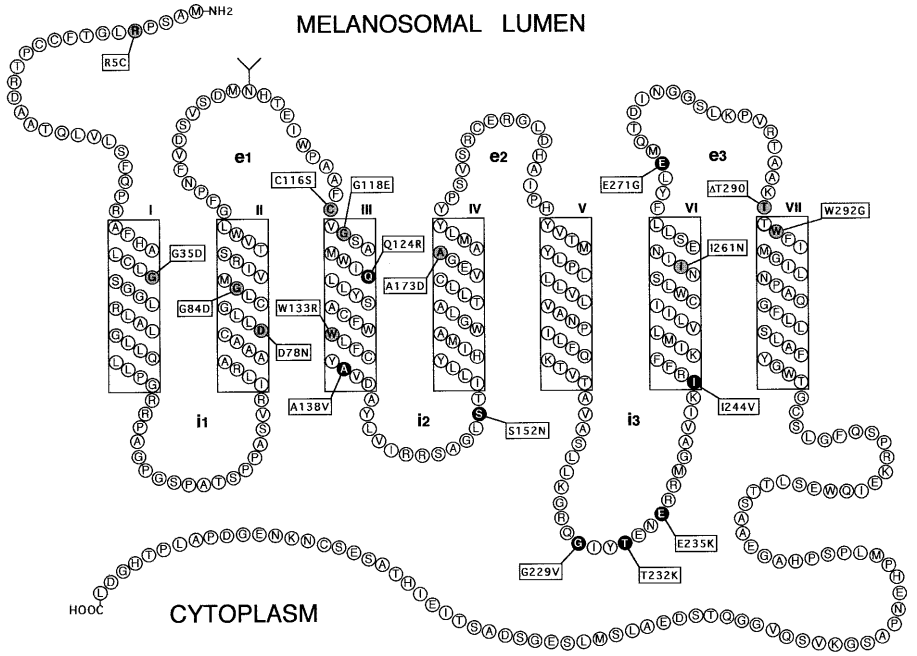

Figure 1. Predicted heptahelical, GPCR-like topology of the OA1 protein. The OA1 protein is inserted within the melanosomal membrane with the N-terminus toward the melanosomal lumen and the C-terminus toward the cytoplasm. Transmembrane α helices are boxed within vertical rectangles and numbered I–VII. Hydrophilic lumenal and intracellular loops are indicated by e_{1-3} and i_{1-3}, respectively (the lumenal loops in OA1 correspond to the extracellular loops in canonical GPCRs). In the first lumenal loop the position of the *N*-glycosylation site is shown. Indicated along the sequence of the OA1 protein are 19 independent missense mutations identified in patients with OA1. Gray circles represent mutations leading to incomplete processing and ER retention of the OA1 protein (group II mutations). Black circles represent mutations that do not hamper the normal processing and targeting of the OA1 protein (group I mutations) and, therefore, may possibly interfere with its proper signaling activity (i.e. with G protein coupling or ligand binding).

some biogenesis (6). More recently, by using several complementary approaches, we found that OA1 shares significant structural and functional similarities with the G protein-coupled receptor (GPCR) superfamily (7). However, in contrast to all other GPCRs identified so far, OA1 is not localized to the plasma membrane, but is instead targeted to intracellular organelles, namely the melanosomes in pigment cells and the lysosomes in transfected non-melanocytic cells. These data indicate that the OA1 protein represents the first example of an exclusively intracellular GPCR, which could act as a 'sensor' of a yet unidentified intra-melanosomal ligand, regulating organelle biogenesis and maturation through activation of heterotrimeric G proteins on the cytoplasmic side of the melanosomal membrane (7).

The addition of OA1 to the long list of GPCR-related diseases represents a major advancement for the study of the pathogenesis of this disorder, first because it provides a possible functional role for the OA1 protein in melanosome biogenesis, but also because of the vast amount of information available on the structural and functional features of the GPCR superfamily. A number of genetic disorders are due to either loss of function or gain of function of different GPCRs. In some cases, the same disorder can be caused by both mechanisms, such as in retinitis pigmentosa (RP), which can be due either to inactivation or to constitutive activation of rhodopsin, the retinal receptor for light (8). The analysis of mutations in patients with OA1 (9,10; M.T Bassi *et al.*, submitted) indicates that ~50% of independent mutations responsible for the disorder are represented by partial or complete deletions of the *OA1* gene, splice site, frameshift and nonsense mutations. All of these mutations are presumably leading to gene inactivation, suggesting that the disease is due to a loss-of-function mechanism. However, there is no obvious functional role for the numerous missense mutations identified along the coding region of the gene (Fig. 1).

In order to shed light into the molecular pathogenesis of OA1 and possibly define critical functional domains within the OA1 protein, we introduced 19 independent missense mutations identified in patients with the disorder within the OA1 cDNA and expressed them in COS-7 cells. The behavior of the different OA1 mutants was compared with that of the wild-type protein by western and immunofluorescence analyses. The results obtained in this study indicate that most missense mutations in ocular albinism determine endoplasmic reticulum (ER) retention of the OA1 protein and suggest that, as in canonical GPCRs, the second and third cytosolic loops of OA1 might be critical for its downstream signaling.

RESULTS

Full glycosylation of OA1 is accomplished in the Golgi apparatus

We have previously reported that the endogenous OA1 protein expressed by normal human melanocytes is detected by western blot mainly as a 60 kDa band, corresponding to the

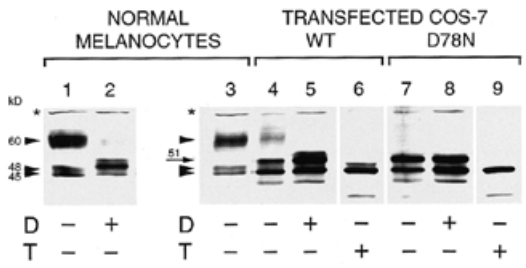

Figure 2. Glycosylation patterns of the wild-type OA1 in normal human melanocytes (lanes 1–3) and of the wild-type and D78N mutant OA1 expressed in COS-7 cells by pRc/RSV vectors (lanes 4–9), in the presence (+) or absence (–) of glycosylation inhibitors. D, deoxymannojirimycin-treated cells; T, tunicamycin-treated cells (melanocytes were not treated with this drug due to its high toxicity for these cells). Arrowheads indicate the 60, 48 and 45 kDa bands corresponding to the endogenous OA1 protein in melanocytes. In particular, the 60 kDa band corresponds to the fully *N*-glycosylated OA1 and the 45–48 kDa doublet to unglycosylated OA1 polypeptides. The arrow indicates the 51 kDa band present in transfected COS-7 cells and corresponding to a partially glycosylated form of OA1, which generates in pre-Golgi compartments and probably accumulates due to overexpression. The asterisks indicate unrelated protein cross-reacting with the anti-OA1 antibody. The additional band visible below the 45 kDa form of OA1 in transfected COS-7 cells probably represents a degradation product of OA1, being absent in mock-transfected cells (Fig. 3).

fully *N*-glycosylated protein. In addition, western analysis reveals the presence of a 45–48 kDa doublet corresponding to unglycosylated OA1 polypeptides (6) (Fig. 2). Since other melanosomal proteins, such as tyrosinase and TRP-1 (11,12) undergo extensive glycosylation in the Golgi apparatus, we tested whether maturation of OA1 also required Golgi processing. Therefore, we treated normal human melanocytes with the α-mannosidase I inhibitor deoxymannojirimycin, which blocks trimming of high-mannose oligosaccharides preventing further modification of N-linked sugars in the Golgi lumen. As shown in Figure 2, treatment with deoxymannojirimycin resulted in disappearance of the 60 kDa band, indicating that full glycosylation of OA1 requires modification of N-linked high-mannose oligosaccharides in the Golgi apparatus. Consistent with these findings, double immunofluorescence assays performed on normal human melanocytes showed that the typical perinuclear accumulation of OA1 colocalizes with the Golgi-resident protein giantin (data not shown).

Similar results were obtained in COS-7 cells transiently transfected with the OA1 expression vector pR/OA1, in which expression of the OA1 cDNA is driven by the strong RSV promoter of the pRc/RSV vector (see Materials and Methods). Indeed, transfected COS-7 cells were able to produce a small amount of the 60 kDa mature form of OA1 (Fig. 2), together with the 45 kDa polypeptide and an additional 51 kDa band (Fig. 2; the 48 kDa band is not evident in transfected COS-7 extracts; see below). As with melanocytes, treatment of the transfected cells with deoxymannojirimycin resulted in the disappearance of the 60 kDa form of OA1 (Fig. 2). Moreover, when transfected COS-7 cells were incubated with tunicamycin, which interferes with N-linked glycosylation in the ER, the 51 kDa protein was also lost (Fig. 2). These findings suggest that the 51 kDa band represents the ER-dependent glycosylated form of OA1, which accumulates in the transfected cells possibly due to overexpression.

In tunicamycin-treated COS-7 cells the anti-OA1 antibody reveals the presence of the 45 kDa band and, to a much lesser extent, a 48 kDa polypeptide, possibly corresponding to that present in melanocytes (Fig. 2). However, the nature of this band in both melanocytes and transfected COS-7 cells remains to be established. Reduction of the fully or partially glycosylated form of OA1 to unglycosylated 45 and 48 kDa polypeptides was also obtained by digesting melanocyte or COS-7 extracts with *N*-glycosidase F (6). Together, these data indicate that in transfected COS-7 cells OA1 follows a processing pathway similar to that of the endogenous protein in melanocytes, being processed to the mature 60 kDa glycoprotein through the Golgi apparatus. However, in COS-7 cells a considerable amount of OA1 remains incompletely glycosylated to a 51 kDa form, possibly due to the high levels of expression obtained by pRc/RSV vectors or to the absence of specific folding factors.

OA1 mutants can be classified into two biochemical groups

Taking advantage of these processing similarities, we performed an *in vitro* study aimed at defining the role of missense mutations identified in patients with ocular albinism. Nineteen independent missense mutations, including a single amino acid deletion (9,10; M.T Bassi *et al.*, submitted) (Table 1 and Fig. 1), were introduced by *in vitro* site-directed mutagenesis into the OA1 cDNA. The resulting mutant cDNAs were then subcloned into the expression vector pRc/RSV similarly to the wild-type cDNA in pR/OA1. On transient transfection of COS-7 cells, the mutant OA1 proteins were characterized with respect to their glycosylation pattern by western blot analysis. This study revealed the presence of essentially two alternative glycosylation patterns (Table 1 and Fig. 3A). When expressed in COS-7 cells ~40% of mutants, including Q124R, A138V, S152N, G229V, T232K, E235K, I244V and E271G (referred to as group I), displayed a western blot pattern completely overlapping that of the wild-type OA1 protein, including the 45, 51 and 60 kDa bands (Fig. 3A).

The remaining 11 mutants (R5C, G35D, D78N, G84D, C116S, G118E, W133R, A173D, I261N, W292G and ΔT290; referred to as group II) showed an altered glycosylation pattern, characterized by the presence of the 45 and 51 kDa bands only and by the absence of the 60 kDa glycosylated form of OA1 (Fig. 3A). The processing of an example of these latter mutants, D78N, is illustrated in Figure 2. The pattern of bands displayed by the mutant is identical in untreated cells or in cells treated with deoxymannojirimycin (Fig. 2), consistent with absence of Golgi processing. In addition, tunicamycin treatment resulted in the production of the 45 kDa unglycosylated protein only (Fig. 2). Thus, group II OA1 mutants appear unable to undergo complete processing in the Golgi, displaying the presence of the 45 kDa unglycosylated polypeptide and of the 51 kDa incompletely glycosylated protein only.

We have previously reported that the OA1 protein appears to follow a lysosomal–melanosomal sorting pathway (7). In fact the endogenous OA1 in melanocytes is localized to the melanosomes, whereas the recombinant protein expressed in non-melanocytic cells such as COS-7 is targeted to the lysosomes (6,7). In order to test for possible sorting defects of the OA1 mutants, we analyzed their subcellular distribution in transfected COS-7 cells by immunofluorescence. As with the

Table 1. Features of missense mutations identified in patients with ocular albinism

OA1 mutant	Position of mutation within the OA1 protein	Processing in COS cells (pRc/RSV vectors)	Subcellular localization in COS cells (pRc/RSV vectors)	Yield obtained in COS cells (LXSN vectors)	Group	References
Wild-type	–	Full glycosylation	Lysosomal	Wild-type	–	–
Q124R	TM III	Full glycosylation	Lysosomal	Wild-type	I	In prep.
A138V	TM III-i2	Full glycosylation	Lysosomal	Wild-type	I	10
S152N	i2	Full glycosylation	Lysosomal	Wild-type	I	10
G229V	i3	Full glycosylation	Lysosomal	Wild-type	I	In prep.
T232K	i3	Full glycosylation	Lysosomal	Wild-type	I	10
E235K	i3	Full glycosylation	Lysosomal	Wild-type	I	10
I244K	i3-TM VI	Full glycosylation	Lysosomal	Wild-type	I	In prep.
E271G	e3	Full glycosylation	Lysosomal	Wild-type	I	In prep.
R5C	N-Ter	Defective glycosylation	ER	Reduced	II	In prep.
G35D	TM I	Defective glycosylation	ER	Reduced	II	9
D78N	TM II	Defective glycosylation	ER	Reduced	II	In prep.
G84D[a]	TM II	Defective glycosylation	ER	Reduced	II	9
C116S[a]	e1-TM III	Defective glycosylation	ER	Reduced	II	In prep.
G118E	TM III	Defective glycosylation	ER	Reduced	II	10
W133R	TM III	Defective glycosylation	ER	Reduced	II	10
A173D	TM IV	Defective glycosylation	ER	Reduced	II	9
I261N	TM VI	Defective glycosylation	ER	Reduced	II	In prep.
DeltaT290	e3-TM VII	Defective glycosylation	ER	Reduced	II	9
W292G[a]	TM VII	Defective glycosylation	ER	Reduced	II	9

[a]At the same positions different substitutions were found, namely G84R, C116R (10) and W292C (M.T. Bassi *et al*., submitted).
TM; transmembrane domain; $e_{1–3}$ and $i_{1–3}$, hydrophilic lumenal and intracellular loops, respectively; in prep., M.T. Bassi *et al*., submitted.

glycosylation patterns, OA1 mutants expressed in COS-7 cells by pRc/RSV vectors displayed two alternative behaviors. Similarly to the wild-type OA1 protein, group I mutants displayed a cytoplasmic vesicular distribution, more concentrated in the perinuclear area and spreading toward the periphery (Fig. 3B, E235K) and co-localized with lysosomal markers (data not shown). In contrast, group II mutants exhibited a fine reticular distribution, with heavy staining of the nuclear membrane, consistent with protein accumulation in the ER (Fig. 3B, A173D). Together, western and immunofluorescence data suggest that group II mutations affect the correct folding of OA1, determining retention of the protein in the ER, thus preventing its proper glycosylation in the Golgi apparatus and its final targeting to the lysosomes.

Group II OA1 mutants accumulate at lower levels in COS-7 cells

Many proteins are subjected to a faster degradation rate when retained in the ER (13). However, we did not notice evident reduction in the yield of group II mutants when transiently expressed in COS-7 cells by pRc/RSV vectors. This could possibly be due to the strong RSV promoter, which leads to the production of large amounts of recombinant proteins in the transfected cells. Alternatively (or in addition) it is possible that, since COS-7 cells are not the endogenous cell type for OA1, they might not possess an efficient degradation machinery for it. In order to verify whether ER-retained mutants were unstable when expressed at lower, more physiological levels, we generated expression constructs for the wild-type and mutant OA1 proteins by introducing the corresponding cDNAs into the retroviral vector LXSN (see Materials and Methods). In this vector, expression of the cloned cDNAs is driven by the retroviral long terminal repeat, a relatively weak promoter compared with RSV.

These constructs were introduced into COS-7 cells by lipofection and the yield of the wild-type and mutant OA1 proteins was analyzed by western blotting. As shown in Figure 4, in these assays, the wild-type OA1 protein was mainly produced as the 45 and 60 kDa bands, with no evident accumulation of the partially glycosylated 51 kDa form. Consistent with the previous results, group I mutants showed the same band pattern and accumulated to levels similar to that of the wild-type OA1 protein (Fig. 4, Q124R to E271G). In contrast, group II mutants not only displayed the absence of the 60 kDa band of OA1, as expected, but also a significant reduction or even absence of the 45 kDa unglycosylated protein (Fig. 4, R5C to W292G). Indeed, these mutants showed western patterns hardly distinguishable from that obtained with the LXSN vector alone (Fig. 4, MOCK).

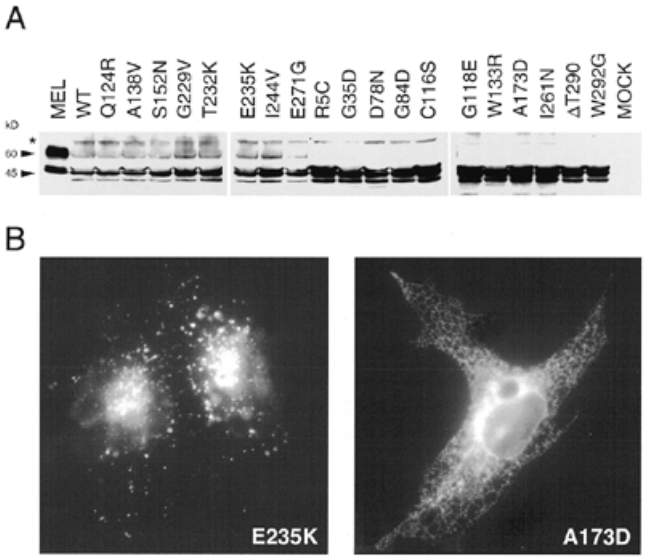

Figure 3. Glycosylation patterns and subcellular distribution of mutant OA1 proteins expressed in COS-7 cells by pRc/RSV vectors. Nineteen OA1 mutants were expressed in COS-7 cells and characterized by western blot (**A**) and immunofluorescence (**B**) analyses performed using the anti-OA1 antiserum. (A) MEL, protein extract from normal human melanocytes; WT, protein extract from COS-7 cells transfected with the pRc/RSV expression vector for the wild-type OA1, displaying the presence of the 60 kDa band and the 51–45 kDa doublet; Q124R to E271G, group I OA1 mutants; R5C to W292G, group II OA1 mutants. COS-7 cells were also transfected with the empty pRc/RSV vector as control (MOCK). The 60 kDa band, corresponding to the fully glycosylated OA1 protein, is present in the wild-type and group I-transefected cells only. The asterisk indicates an unrelated protein cross-reacting with the anti-OA1 antibody. The additional band visible below the 45 kDa form of OA1 in transfected COS-7 cells probably represents a degradation product of OA1, being absent in mock-transfected cells. (B) Representative examples of immunofluorescence patterns observed for mutants belonging to groups I (E235K) and II (A173D). The immunofluorescence pattern of mutant E235K is characterized by a vesicular cytoplasmic distribution resembling that of the wild-type, whereas the A173D mutant displays a fine reticular distribution consistent with protein accumulation in the ER. All mutants belonging to the same group show similar patterns.

The endogenous D78N mutant is undetectable in patient melanocytes

In order to test for the actual behavior of group II OA1 mutants when endogenously expressed in pigment cells, we isolated and analyzed skin melanocytes from a patient affected with ocular albinism and carrying the D78N mutation (Fig. 5). Despite the difficulties of growing these cells *in vitro*, we could obtain sufficient material for immunofluorescence and western analyses with the anti-OA1 antibody (Fig. 5). In the patient melanocytes we could not detect any OA1 protein by immunofluorescence (Fig. 5A, D78N). Western blot analysis revealed the presence of very weak bands, hardly distinguishable from the background and possibly corresponding to unglycosylated or partially glycosylated OA1 polypeptides (Fig. 5B, D78N). These findings suggest that group II OA1 mutants endogenously expressed in melanocytes behave as the recombinant proteins expressed in COS-7 cells, being retained in the ER and then presumably degraded, with resulting absence or major reduction of OA1 protein.

DISCUSSION

In the attempt to define the molecular mechanisms underlying OA1 and to identify possible functional domains within the OA1 protein, we have characterized 19 different missense mutations essentially corresponding to the complete spectrum of missense mutations identified so far in patients with the disorder (9,10; M.T Bassi *et al*., submitted). We took advantage of the finding that the endogenous OA1 protein in melanocytes and the recombinant OA1 protein in COS-7 cells appear to follow similar processing and sorting pathways, being glycosylated in the Golgi apparatus and sorted along the lysosomal–melanosomal pathway, similarly to other melanosomal proteins such as tyrosinase and TRP-1 (11,12,14). Based on this correspondence, we performed an *in vitro* study aimed at defining the possible effects of missense mutations identified in patients with ocular albinism on the processing and subcellular distribution of the OA1 protein. The results of this study are summarized in Table 1.

Our analysis indicates the presence of at least two distinct mechanisms by which the function of OA1 can be disturbed by missense mutations: one is represented by retention of the protein in the ER, whereas the other remains less defined, possibly relating to the signaling activity of OA1 as a GPCR. In fact, based on immunoblot and immunofluorescence patterns, OA1 mutants could schematically be subdivided into two groups. Group I mutants (Table 1) display processing and sorting behaviors indistinguishable from that of the wild-type OA1, suggesting that mutations belonging to this group could affect residues that are important for the signaling activity of the protein. In contrast, group II mutants (Table 1) showed an altered glycosylation pattern, characterized by the absence of

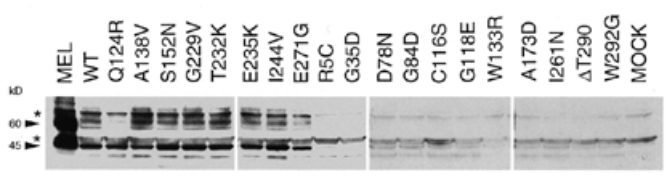

Figure 4. Western blot patterns displayed by mutant OA1 proteins expressed in COS-7 cells by LXSN vectors. MEL, protein extract from normal human melanocytes; WT, protein extract from COS-7 cells transfected with the LXSN expression vector for the wild-type OA1, displaying the presence of the 45 and 60 kDa bands; Q124R to E271G, group I OA1 mutants; R5C to W292G, group II OA1 mutants. COS-7 cells were also transfected with the empty LXSN vector as control (MOCK). The 45 and 60 kDa bands of OA1 are clearly present in the wild-type and group I-transfected cells only. In both melanocytes and transfected COS-7 cells, the fully glycosylated form of OA1 usually appears as a thick and diffused 60 kDa band, although sometimes it can also present as a doublet, as in this case. The amount of glycosylated OA1 appears slightly different from experiment to experiment, possibly depending on the variation intrinsic to transient transfections. The asterisks indicate unrelated proteins cross-reacting with the anti-OA1 antibody (these are particularly evident here due to the high amounts of protein loaded and to the long exposure time used). The additional band visible below the 45 kDa form of OA1 in transfected COS-7 cells (and weakly visible also in melanocytes) probably represents a degradation product of OA1, being absent in mock-transfected

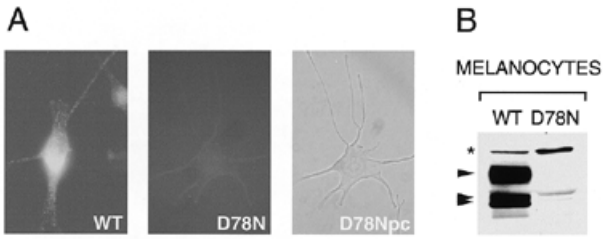

Figure 5. Immunofluorescence (**A**) and western blot analysis (**B**) of normal human melanocytes (WT) and melanocytes isolated from a patient with ocular albinism carrying the D78N mutation (D78N), performed using the anti-OA1 antiserum. (A) Phase contrast and immunofluorescence imaging of wild-type and D78N melanocytes. D78Npc, phase contrast image of the same cell analyzed by immunofluorescence in D78N. (B) Western blot analysis of wild-type and D78N melanocytes. WT and D78N, protein extracts from wild-type or patient melanocytes subjected to immunoblotting with the anti-OA1 antibody. In the patient melanocytes not only the 60 kDa but also the lower molecular weight bands of OA1 are essentially undetectable (arrowheads), similarly to the results observed in COS-7 cells using LXSN vectors. The asterisk indicates an unrelated protein cross-reacting with the anti-OA1 antibody. The additional band weakly visible below the 45 kDa form of OA1 in wild-type melanocytes probably represents a degradation product of OA1, being absent in the patient cells.

the fully glycosylated 60 kDa form of OA1, and exhibited a fine reticular distribution with heavy staining of the nuclear membrane, consistent with protein accumulation in the ER. Mutations belonging to group II appear to cause the retention of the OA1 protein in the ER, thus preventing both its proper glycosylation in the Golgi apparatus and its final targeting to the lysosomes. The sorting (and the folding) of OA1 in transfected COS-7 cells did not appear to be affected by glycosylation itself, since use of tunicamycin during transfections did not modify its subcellular localization (data not shown). In this respect, OA1 behaves differently from lysosomal hydrolases and similarly to classical lysosomal membrane proteins (15).

Interestingly, although missense mutations are spread along the entire length of the OA1 protein, with the exception of the C-terminal tail, group II mutations are mainly concentrated within (or close to) the putative transmembrane domains (Table 1 and Fig. 1). In several cases they are represented by introduction or removal of charged residues (G35D, D78N, G84D, G118E, W133R and A173D). It is reasonable to suppose that these substitutions affect the energetics of packing of hydrophobic α helices. Moreover, some group II mutations, namely D78N and C116S, affect amino acids that are highly conserved in most GPCRs and are known to be important for the receptor's folding and structure. In particular, whereas the role of the aspartic acid in TM2 has not been precisely defined, the cysteine residue in the first extracellular loop is known to participate in an intramolecular disulfide bridge with its partner cysteine in the second extracellular loop (16) (Fig. 1). Mutations at residues equivalent to D78 and C116 in canonical GPCRs have been found to be responsible for other genetic disorders and to determine receptor instability, misfolding and misfunctioning (16–18). It is conceivable that group II mutations affect residues with an important structural role and therefore interfere with the correct folding and insertion of OA1 across the membrane, with resulting retention of the protein by the ER quality control (13).

The final defect displayed by group II mutants in both COS-7 cells and melanocytes does not appear to be limited to their inability to reach the correct subcellular location (the lysosomes in COS-7 cells and the melanosomes in melanocytes). Indeed, the use of low expression levels in COS-7 cells revealed that these mutants also accumulate at much lower levels with respect to the wild-type OA1. The relevance of these latter findings for the actual role of group II mutations in the pathogenesis of ocular albinism is confirmed by the analysis of melanocytes isolated from a patient carrying the D78N mutation. Although this is the only patient with a missense mutation from whom we could obtain a skin biopsy, the lack of detectable OA1 protein in his cultured melanocytes strongly suggests that the same might occur for the other group II mutants when endogenously expressed *in vivo*. Our findings are in agreement with the current view regarding the management of unfolded proteins by the ER quality control (19) and in particular suggest that ER retention of group II OA1 mutants, endogenously expressed in the pigment cells of patients with ocular albinism, is associated with their degradation. Thus, a pure loss-of-function mechanism, with lack or major reduction of OA1 protein, appears to be responsible for ocular albinism in the presence not only of clearly inactivating mutations but also of most missense mutations.

In addition, to be relevant for the pathogenesis of OA1, these results might also be significant with respect to its molecular diagnosis, which could be obtained by monitoring the presence or absence of OA1 protein in skin biopsies, and with respect to gene therapy. Group II mutations represent ~60% of all independent missense mutations identified so far in patients with this disorder, suggesting that the OA1 protein is particularly prone to misfold or sensitive to the ER quality control, possibly due to its membrane topology. A similar behavior has been reported for a number of integral membrane proteins, including other GPCRs, such as rhodopsin (20), as well as

unrelated multispanning membrane proteins involved in genetic disorders, such as CFTR and PLP (21,22). As with OA1, many mutations leading to protein misfolding and ER retention of these proteins cluster within the putative transmembrane domains (20,22,23). Our results with OA1 mutants join OA1 to the growing list of disorders due to protein folding defects and corroborate the concept that protein misfolding represents a common and crucial theme in human pathology.

The functional role of group I mutations remains more difficult to interpretation. These mutations displayed western blot and immunofluorescence patterns completely overlapping that of the wild-type OA1 protein on expression in COS-7 cells. In addition, preliminary immunofluorescence experiments indicate that these mutants behave as the wild-type also when expressed in mouse melanocytes. These data suggest that group I mutants might be defective in the signaling activity of OA1 as a GPCR. At present it is difficult to precisely predict which could be the specific role of the mutated residues, due to the low sequence similarities between OA1 and best characterized GPCRs. However, significantly enough, most (6 of 8) of group I mutations are located within or very close to the putative second and third intracellular loops of the OA1 protein, two regions that in canonical GPCRs are known to play a critical role particularly in their downstream signaling (24) (Table 1 and Fig. 1). Naturally occurring or experimentally created mutations at these locations have been shown to interfere with several functional properties of these receptors, including agonist affinity, G protein coupling, activation of downstream effectors and agonist-promoted desensitization (http://mgddk1.niddk.nih.gov:8000/MutationAnalysis.html). Thus, it is conceivable that the second and third intracellular loops might represent important functional domains in OA1 as they do in canonical GPCRs.

In contrast to other GPCR-related diseases, such as RP (8), in OA1 there is no evidence of phenotype–genotype correlation, with the major ocular features being invariably present in all affected patients with comparable severity. This suggests that, whatever their nature, all *OA1* gene mutations should have an equivalent effect on the OA1 protein function. Given that (i) ~50% of independent mutations identified in patients with ocular albinism are clearly leading to gene inactivation, determining absence or premature truncation of the OA1 protein; and (ii) group II missense mutations (representing 60% of all missense mutations) appear to cause a major reduction or complete deficiency of OA1 protein, it is conceivable that group I missense mutations also determine loss of function of OA1. Preliminary experiments of co-immunoprecipitation and recombinant protein pull-down (using recombinant proteins obtained by fusing *Schistosoma japonicum* glutathione *S*-transferase with the wild-type or mutant third cytoplasmic loop of OA1) did not reveal appreciable differences in G protein-binding ability between group I mutants and the wild-type OA1. Further studies, in particular based on the knowledge of the OA1 agonist(s) and downstream effector(s), will be necessary to precisely define the biochemical defect determined by group I mutations on the signaling activity of the OA1 receptor in response to ligand binding.

In conclusion, we utilized a biochemical and morphological approach to characterize the entire spectrum of independent missense mutations described so far in patients with OA1. Our study shed light on the molecular bases of a relevant percentage of OA1 and in the meantime emphasized the importance of particular residues or regions for the correct structure and/or function of the OA1 protein. As with similar studies previously performed for rhodopsin and other GPCRs, which gave considerable insights into the structure–function relationships and allowed subsequent more detailed characterization of receptors (18,20,25), our analysis represents the first step toward the molecular dissection of OA1 and of its role in physiology and disease.

MATERIALS AND METHODS

Expression constructs and mutagenesis

Site-directed *in vitro* mutagenesis was performed on the OA1 cDNA, cloned into the *Eco*RI and *Sac*II sites of pBluescript SK$^-$ (Stratagene, La Jolla, CA), by using the Transformer Site-Directed Mutagenesis kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Wild-type and mutant cDNAs were subsequently excised by *Eco*RI and *Xmn*I digestion and subcloned into the blunted *Bst*XI site of the pRc/RSV expression vector (Invitrogen, Carlsbad, CA) and into the *Eco*RI and blunted *Xho*I sites of the LXSN retroviral vector (26). The nucleotide sequence of all mutant constructs, either in pBluescript or in pRc/RSV and LXSN vectors, was verified by DNA sequencing. In order to avoid the possibility that the presence of any small rearrangement far away from the mutation site could compromise the results, two independent mutant cDNAs for each mutation were selected, subcloned into the expression pRc/RSV vector and subjected to western and immunofluorescence analyses. To introduce the 19 mutations in the OA1 cDNA the following primers were used:
selection primer, 5′-CTGGTGAGTATTCAACCAAGTC-3′;
R5C, 5′-GGCCTCCCCGTGCCTAGGGAC-3′;
G35D, 5′-CTCTGCCTGGACAGCGGCGG-3′;
D78N, 5′-CGCTGCCTGCAACCTTCTCGG-3′;
G84D, 5′-GGCTGCCTGGATATGGTGATC-3′;
C116S, 5′-GCTGCTTTCTCCGTGGGGAGTG-3′;
G118E, 5′-CTTTCTGCGTGGAGAGTGCGATG-3′;
Q124R, 5′-GATGTGGATCCGGCTGTTGTAC-3′;
W133R, 5′-CTGCTTCTGGAGGCTGTTTTGC-3′;
A138V, 5′-GTTTTGCTATGTAGTGGATGCTTA-3′;
S152N, 5′-GCAGGACTGAACACCATCCTG-3′;
A173D, 5′-GTGGAGGGAGACGCCATGCTC-3′;
G229V, 5′-GGAAGACAAGTCATTTACACGG-3′;
T232K, 5′-AGGCATTTACAAGGAGAACGAG-3′;
E235K, 5′-CACGGAGAACAAGAGGAGGATG-3′;
I244V, 5′-CCGTGATCAAGGTCCGATTTTTC-3′;
I261N, 5′-GTTGTCGAATAACATCAATGAAAG-3′;
E271G, 5′-TATTCTATCTTGGGATGCAAACAG-3′;
ΔT290, 5′-CTGCAGCCAAGACATGGTTTATTATG-3′;
W292G, 5′-CAAGACCACAGGGTTTATTATGG-3′.

Cell culture and transfection

Normal and patient melanocytes were isolated and cultured as described (6,27). COS-7 cells were grown as described (6) and transiently transfected either (i) by electroporation, using the purified (Plasmid kit; Qiagen, Valencia, CA) expression vectors pRc/RSV and grown 24–48 h before fixation or harvesting; or (ii) with the Lipofectamine reagent (Gibco BRL, Gaithersburg, MD), using the expression vector LXSN and grown 60 h before harvesting. The results presented are representative of at least two independent transfection experiments. In addition, all constructs were checked for the same

DNA concentration by both spectrophotometer and agarose gel analyses and in the case of LXSN vectors, for the same expression levels in transfected cells by northern blotting. For glycosylation analysis, melanocytes were treated with deoxymannojirimycin at 5 mM for 4 days, whereas transfected COS-7 cells were treated either with deoxymannojirimycin at 5 mM or with tunicamycin at 2 µg/ml during the entire transfection time.

Western immunoblotting and immunofluorescence analyses

Western and immunofluorescence analyses were performed essentially as described (6,7). Approximately 40 µg of protein extracts from melanocytes and 70 or 140 µg of protein extracts from COS-7 transfected with pRc/RSV or LXSN vectors, respectively, were separated on SDS–polyacrylamide gels (7.5–9%) and transferred to PVDF membrane sheets (Hybond-P; Amersham Pharmacia Biotech, Indianapolis, IN) using the Mini-Protean and the Mini Trans-Blot apparatus (BioRad, Hercules, CA). Visualization of antibody binding was carried out with Enhanced Chemiluminescence Plus (Amersham Pharmacia Biotech) according to the manufacturer's instructions. For immunofluorescence analysis, melanocytes or transfected COS-7 cells were fixed with methanol at –20°C. Affinity-purified anti-OA1 antibodies W7 were previously described (6) and used at 0.5 and 1.5 µg/ml for western and immunofluorescence analyses, respectively.

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